

**METAL IONS AS
NUCLEAR MAGNETIC RESONANCE PROBES
IN BIOLOGY**

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Lund 1978

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LUND 1978



Dokumentutgivare
University of Lund, Dept. of Physical
Handläggare Chemistry

Dokumentnamn
Dissertation
Utgivningsdatum
Nov 1978

Dokumentbeteckning
Ärendebeteckning

Författare
Dennis R. Burton

Dokumenttitel och undertitel

Metal Ions as Nuclear Magnetic Resonance Probes in Biology.

Referat (sammandrag)

The Proton Relaxation Enhancement (PRE) Method applied to complexes of Gd(III) with antibody and antibody fragments illustrated some of the strengths of and difficulties in the method. A review on PRE critically examined the literature. A new method - the Proton/Deuteron Relaxation Enhancement Method - was proposed to avoid many of the difficulties of conventional PRE. The PRE studies on antibodies implied considerable internal mobility in the IgG molecule. PRE studies on pig antibodies in the presence of antigen provided evidence against the conformational change theory of complement triggering. NMR and complement activation studies of the interaction of protein A and one of its fragments with Fc fragment suggested cross-linking of Fc to give protein A its complement-activating ability.

^{23}Na NMR was used to probe ion binding to chromatin and to investigate the interaction of polyamines and DNA. The melting of DNA and transfer RNA was studied by ^{23}Na , ^{25}Mg and ^{43}Ca NMR. The role of metal ion NMR in investigating polynucleotide structure and function was discussed.

Referat skrivet av
author

Förslag till ytterligare nyckelord

Klassifikationssystem och -klass(er)

Indextermer (ange källa)

Omfång

Övriga bibliografiska uppgifter

Språk
English

Sekretessuppgifter

ISSN

ISBN

Dokumentet kan erhållas från
D.R. Burton, Physical Chemistry 2,
Chemical Centre, 220 07 LUND 7, Sweden

Mottagarens uppgifter

Pris

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ACKNOWLEDGEMENTS

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INTRODUCTION

For many biophysicists, NMR in biology means high resolution proton NMR. Even with the introduction of the very high magnetic fields of the superconducting magnet, only relatively small macromolecules can generally be studied in this way. For larger proteins e.g. antibodies and polynucleotides e.g. DNA another approach must be found. A general and useful approach has been to look either directly or indirectly at small molecules or ions in interaction with a macromolecule, so called probes. Nature provides us with probes in many macromolecular systems in the form of metal ions e.g. metallo-proteins, metal ions interacting with the phosphate groups of DNA and RNA. The metal ions in these examples are of course interesting not only as probes of the macromolecular structure to which they are bound but also in themselves as an integral part of the biological macromolecule. If the system to be studied does not involve a suitable metal ion it is often possible to introduce one without serious perturbation of structure or function. In this latter case the metal ion is of interest simply as a reporter on macromolecular structure.

This thesis centres largely around the use, in two very different ways, of metal ions as NMR probes in biological molecules.

The first approach involves the application of paramagnetic ions as probes of macromolecular structure and dynamics. A large body of the work is concerned with the use of the paramagnetic ion, Gd(III), in the study of the antibody molecule and its interaction with antigen. The ion does not occur in nature in combination with antibody but its presence would not seem to have any major perturbatory effects⁽¹⁾. Information on the environment of the paramagnetic ion, in particular molecular motion information, is obtained through its relaxing effect on solvent water

protons - the so called Proton Relaxation Enhancement (PRE) technique. The PRE/antibody work is found in papers I - VI:

- I "Difficulties in determining accurate molecular motion parameters from PRE measurements as illustrated by the IgG · Gd(III) system". D.R. Burton, S. Forsén, G. Karlström, R.A. Dwek, A.C. McLaughlin and S. Wain-Hobson (1976) Eur. J. Biochem 71, 519.
- II "The determination of molecular motion parameters from PRE measurements in a number of Gd(III) · antibody fragment complexes". Ibid. (1977) Eur. J. Biochem. 75, 445.
- III "A Novel Approach to Water Proton Relaxation in Paramagnetic Ion · Macromolecule Complexes". D.R. Burton, R.A. Dwek, S. Forsén and G. Karlström (1977) Biochemistry, 16, 250.
- IV "The interaction of protein A and Fc fragment of rabbit IgG as probed by complement fixation and NMR studies". C. Wright, K.J. Willan, J. Sjö Dahl, D.R. Burton and R.A. Dwek (1977) Biochem. J. 167, 661.
- V "Proton Relaxation Enhancement to Probe the Effects of Antigen Binding on Pig Antibody Structure". D.R. Burton, S. Forsén, F. Franěk and J. Novotný (1978), to be published.
- VI "Proton Relaxation Enhancement in Biochemistry: A Critical Survey". D.R. Burton, S. Forsén, G. Karlström and R.A. Dwek (1979) Progress in NMR Spectroscopy, to be published.

The PRE/antibody work has two main themes:

- (1) a better understanding of the PRE technique, particularly its limitations. Paper VI, a review article on PRE is the culmination of work along these lines. Paper III presents a novel approach to water relaxation in paramagnetic ion • macromolecule complexes, avoiding many of the inherent difficulties of the conventional PRE method.
- (2) the mechanism of complement binding to antibody in the presence of antigen. Papers I to III on antibody alone can be seen as providing the groundwork for complement "trigger" studies and even suggest a possible mechanism. Paper V is a study in the presence of antigen. Paper IV on protein A differs somewhat in its approach from the rest of the antibody work but is important for its conclusions with respect to complement binding.

The second approach to the use of metal ions as NMR probes is to look directly at the NMR signals of the metal ions involved. A number of biologically very significant metal ions e.g. $^{23}\text{Na}^+$, $^{25}\text{Mg}^{2+}$, $^{43}\text{Ca}^{2+}$ are open to study in this way. The nuclei mentioned all have spin $> 1/2$ and are thus quadrupolar. The type of information provided and the way this information is obtained will therefore differ somewhat from that found to be the case for spin- $1/2$ nuclei such as protons. The use of the metal ion signal however as a titration indicator in binding studies is straightforward.

Metal ions are positively charged and therefore often found in association with negatively charged species. One of the most negatively charged types of biological molecule is the polynucleotide e.g. DNA, transfer RNA. Both of these molecules are heavily dependent for stability on their interactions with metal ions. In the case of DNA in the eucaryotic cell much of the negative charge of the phosphate groups is neutralised

by basic proteins known as histones leading to the building of the chromatin complex. Some 40% however is left over to metal ions such as Na^+ . In paper VII we ask ^{23}Na NMR to answer the question - is there any difference in the nature of the Na^+ binding sites in DNA and chromatin?

VII " ^{23}Na NMR as a probe of ion binding to chromatin".
D.R. Burton and P. Reimarsson (1978). FEBS Letts
89, 183.

Later in the thesis some new unpublished data on the interaction of polyamines and DNA using ^{23}Na NMR as a titration indicator is presented. The melting of DNA and transfer RNA studied by ^{23}Na , ^{25}Mg and ^{43}Ca NMR is also briefly considered. Finally the potential of metal ion NMR in the study of polynucleotide structure and function is discussed and it is claimed that this could be a powerful method in the future.

THE PROTON RELAXATION ENHANCEMENT (PRE) METHOD

The theory of the PRE method is described both schematically and in detail in the review (paper VI). We shall not repeat that material here but shall rather confine ourselves to the briefest of descriptions followed by a few reflections on the usefulness of the method in biochemistry.

The addition of a paramagnetic ion to water produces an increase in the solvent water proton relaxation rates by virtue of the large electronic magnetic moment associated with the unpaired electrons of the paramagnetic ion. The further addition of a macromolecule to which the paramagnetic ion binds can produce a further increase in water proton relaxation rates by virtue of correlation time changes in the presence of macromolecule. The observation and analysis of this second relaxation enhancement is the essence of the

PRE method. As the observed enhancement is proportional to the amount of metal bound to macromolecule, PRE can be simply used as a titration indicator to yield metal binding information. It can similarly be used in an empirical way to attempt to detect changes when some parameter of the system is changed e.g. the binding of antigen to antibody in paper V.

The magnitude of the PRE effect has been widely analysed in terms of a number of equations collectively known as the Solomon-Bloembergen-Morgan (SBM) equations⁽²⁻⁶⁾ after their authors. The equations contain the following variables which therefore determine the magnitude of observed enhancements:

B, τ_V a constant and a correlation time determining the electron spin relaxation time of the paramagnetic ion, τ_S .

τ_R rotational correlation time for the ion . macromolecule complex.

τ_M lifetime of a water molecule in the metal ion coordination sphere.

q number of exchangeable water molecules in the metal ion coordination sphere (often loosely equated with coordination number).

r metal ion - water proton distance.

E_V, E_R, E_M activation energies corresponding to the correlation times.

T temperature.

ω NMR frequency.

Of these variables, temperature and frequency are known and can be varied experimentally. The favoured approach to the quantitative application of PRE has thus been to obtain relaxation data at varying temperature and frequency and best-fit the data using the above parameters as variables in the SBM equations. In view of the large number of parameters (up to 9) involved in this multi-parameter fitting procedure considerable problems in obtaining good definition of these parameters can arise. Papers I and II highlight these problems in complexes of Gd(III) with antibody (rabbit IgG) and antibody fragments. Most dramatically we found that, despite the collection of extensive relaxation data, we were unable to define the parameter q for the IgG · Gd(III) complex better than to a value within the limits 2 - 8.

For the IgG · Gd(III) complex we were particularly interested in the rotational correlation time τ_R (see later) but were unable to define this parameter satisfactorily by conventional PRE. To do this it was necessary to develop a new method - the Proton Relaxation Enhancement/Deuteron Relaxation Enhancement (PRE/DRE) Method - which is presented in paper III. The basis of the method is the comparison of solvent water proton and deuteron relaxation rates in a macromolecule/paramagnetic ion/H₂O/D₂O solution at a limited number of magnetic fields (four in our case). In the analysis of the results, exact solution of equations replaces the multi-parameter best-fitting procedure of conventional PRE resulting in a much better definition of parameters and a considerable saving of time and energy. The method does have a number of limitations; these along with the advantages of the method are discussed in detail in the review (paper VI).

At this point we can also mention another 'special' method which we made use of in paper II. This is Navon's method⁽⁷⁾ for the determination of q from a single T_1 and T_2 measurement which is discussed at some length in the review. Generally we have tried in the review to emphasize the value of special

methods such as Navon's and the PRE/DRE method which circumvent the problems associated with (a) multi-parameter fitting and (b) assuming an equation for the functional form of the electron spin relaxation time, τ_S .

Papers I and II show the considerable difficulties we encountered in attempting to extract parameters such as correlation times and coordination numbers from extensive PRE data by multi-parameter fitting. PRE has been widely used in biochemistry: correlation times derived from PRE are often used in distance calculations and coordination numbers are of great interest as regards functional properties of biomolecules e.g. carbonic anhydrase. However the difficulties associated with multi-parameter fitting have not generally been realised as documented in a case-by-case examination of the PRE literature in the review (paper VI). The review shows that many of the conclusions drawn from PRE results are open to debate and that greater care and awareness of the problems in its application are necessary if PRE is to make a positive contribution to the solution of problems in biology.

ANTIBODIES, COMPLEMENT TRIGGERING AND PRE

Immune defence

During his lifetime, the average person will be in contact with some quite unpleasant molecules. Many of these molecules will be taken care of by an amazingly complex and efficient system known as the immune defence system. In one of the principal pathways of this system it is the antibody molecule which recognises and marks the invading agent (the antigen) for destruction and a sequence of proteins referred to as complement which does the destroying. We can visualise the events occurring in a simplified schematic manner as shown below:

Foreign agent (Antigen)
enters the body



Antigen recognised by Antibody
Formation of Antigen-Antibody complexes
(aggregates)



These complexes "trigger" the
Complement Sequence leading to
destruction of the invading agent
e.g. by cell lysis

Antibody structure

The principal antibody in the serum is immunoglobulin G (IgG) which has a molecular weight of about 150,000 and a domain structure as shown in Fig. 1. There are 12 domains with each having a molecular weight of about 12,500⁽⁸⁾.

The antigen binding sites are found on the Fab (Fab = fraction antigen binding) arms of the molecule. The structure of the antigen binding site regions determines the specificity of a given antibody molecule and the possibility of varying this structure while essentially keeping the rest of the molecule intact accounts for the great diversity of antibodies. The molecule is divalent with respect to antigen allowing one molecule to cross-link different antigen molecules and in the case of multivalent antigens (i.e. those with more than one antigenic group or determinant) build aggregates. The flexibility of the Fab arms about the hinge region joining them to the Fc region may facilitate aggregation with multivalent antigens by allowing some leeway in the distance between antigenic determinants

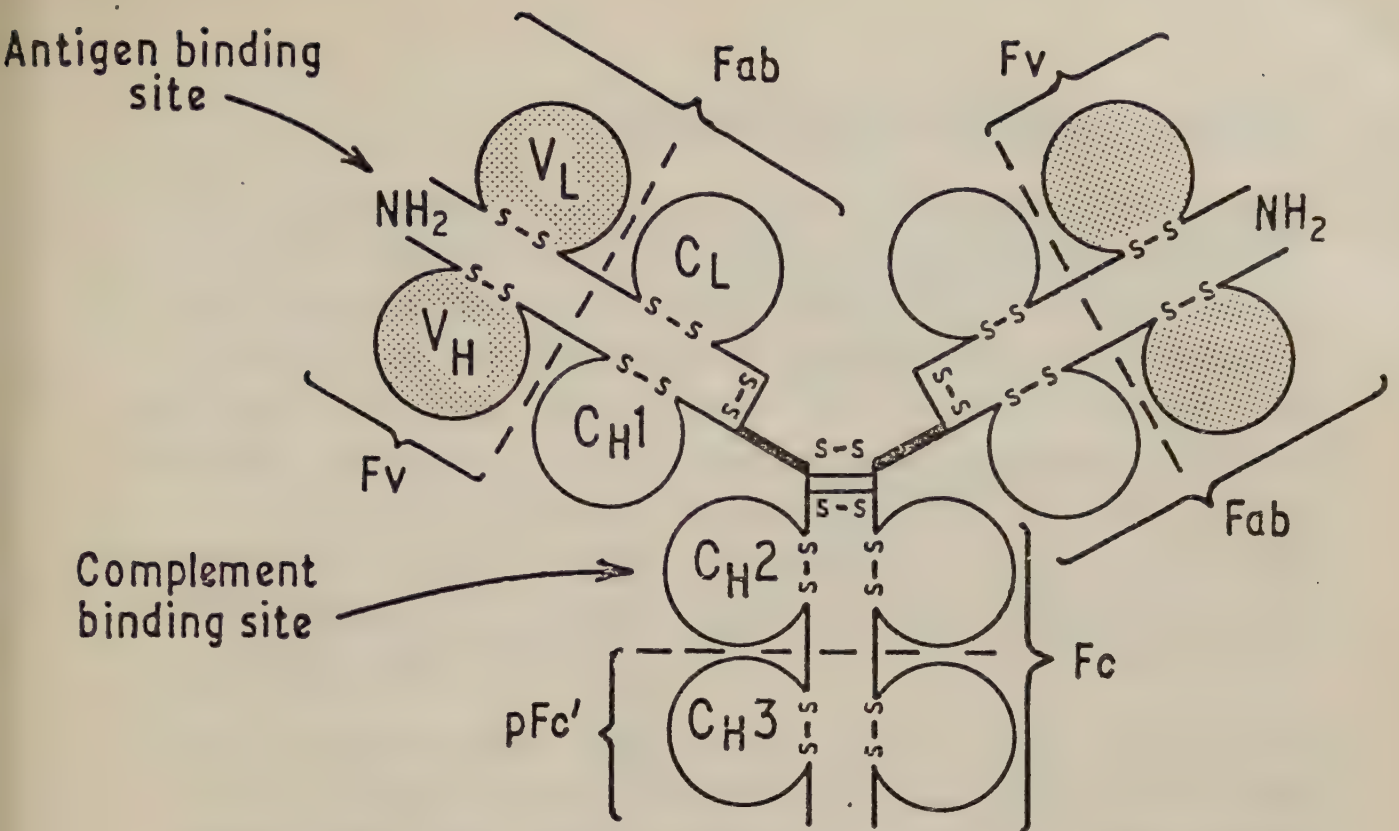


Fig. 1. Structure of IgG

The different domains are shown by circled regions where V signifies variable domain, C constant domain, H belonging to the heavy chain and L belonging to the light chain. The various proteolytic fragments Fc, pFc', Fab and Fv are also indicated. The antigen and complement binding sites are approximately 7.5 nm apart.

required for successful complex formation.

In the Fc (Fc = fraction crystalline) region of the antibody are found the two equivalent complement binding sites in the C_H2 domains and the monocyte binding sites in the C_H3 domains. It is to the complement binding site that the first protein of the complement sequence binds on the formation of specific antibody - antigen complexes. We shall now discuss "complement" in a little more detail.

Complement and Complement Triggering

Complement (see e.g.^(9, 10)) is a system or sequence of 11 proteins designated by the letter C and a number; C1, C2, C3 and so on. The proteins early in the sequence activate those coming later with one molecule serving to activate many molecules of the next stage in the sequence to give the process a cascade nature (compare blood clotting). The proteins later in the sequence are responsible for the destruction of the invading agent e.g. by "punching" holes in its cell membrane leading to cell lysis. The first protein in the sequence, C1, is involved in binding to antibody.

C1 is actually an assembly of subunits designated Clq,Clr and Cls with the assembly consisting of one molecule of subunit Clq, two of Clr and four of Cls. The subunits are held together by non-covalent forces with Ca^{2+} ions playing a critical role here. Clq (molecular weight 400,000) is the recognition unit of the complement system being hexavalent in its site for binding to antibody. For Clq to bind to IgG, at least two IgG molecules occupying adjacent sites on the cell surface of the invading agent (antigen) are required. The situation arising is visualised in Fig. 2⁽¹¹⁾.

It is the binding of Clq to antibody aggregates which sets in motion the complement sequence. The recognition of antibody aggregates by Clq is referred to as complement triggering (reviewed⁽¹²⁾). Triggering only occurs in the presence of antigen i.e. Clq is only induced to bind to antibody when antibody is in interaction with antigen - clearly it would be a disastrous state of affairs if complement was permanently switched on. And yet the Clq and antigen binding sites are approximately 7.5 nm apart. How is binding to one site linked to the other - how does triggering occur?

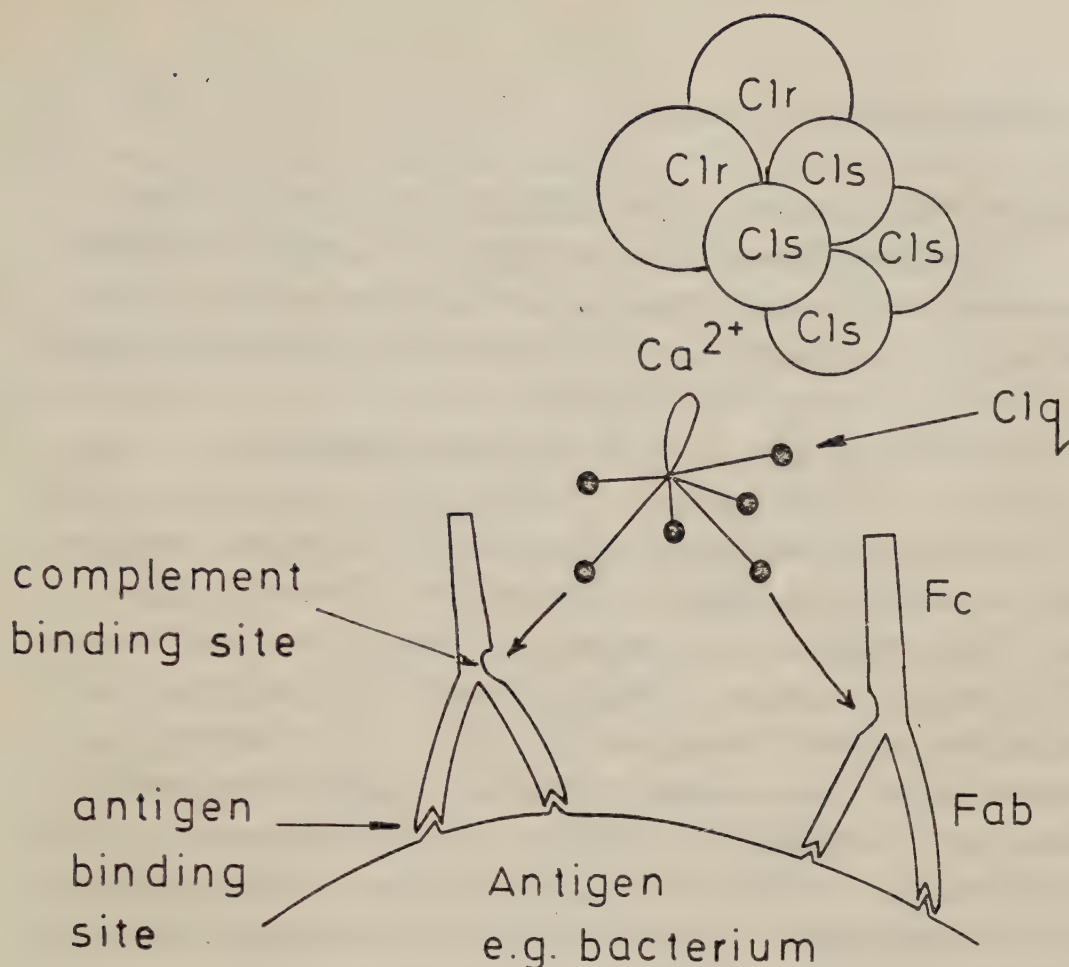


Fig. 2. A schematic representation of the binding of complement factor C1 to two adjacent IgG molecules on the cell surface of an antigen.

Two principal theories have been advanced:

- 1) Conformational change theory. Antigen binding to the Fab region of the antibody molecule causes structural changes in the Fc region leading to the generation of C1q binding sites.
- 2) Aggregation theory. Recognising (a) that at least 2 IgG molecules in close proximity are required for complement activation and (b) the multivalent nature of C1q, this theory suggests that the close proximity of Fc regions is the critical factor.

The experimental approach to investigating the mechanism of complement triggering has largely centered around proving or disproving theory (1) i.e. attempting to detect changes in antibody structure on antigen binding.

PRE and Antibodies

Where does PRE come into this discussion about antibody structure and complement triggering? The answer is that we have used the method to study antibodies alone and in the presence of antigen with implications for the mechanism of complement triggering. We shall begin with a consideration of the PRE of the Gd(III)·antibody complex.

Using PRE as a titration indicator as referred to earlier, studies of non-immune rabbit IgG and the fragments Fc, pFc' and Fab (see Fig. 1) located two equivalent tight Gd(III) binding sites on the C_H3 domains of IgG with weaker sites in the Fab region^(13, 1)*. Experimental conditions were thus easily arranged so that observed PRE effects in the IgG·Gd(III) complex arose almost exclusively from the Gd(III) ions in the C_H3 domains. A quantitative analysis of PRE data for the IgG·Gd(III) complex (papers I and III) then yields information on the environment of these metals ions.

The most interesting feature with respect to antibody structure, or more correctly dynamics, to emerge from the analysis was a rotational correlation time (τ_R) more than an order of magnitude shorter than observed by other methods^(14, 15) for the tumbling motion of the whole IgG molecule. The τ_R value found was interpreted as indicating considerable internal motion in the C_H3 domains. This was an interesting conclusion in view of the almost simultaneous finding from crystallographic studies of considerable motional freedom in the Fc region of an IgG molecule⁽¹⁶⁾. In that paper the authors proposed that this motion may be lost on antigen binding and that the trigger of complement activation would therefore be a flexible → rigid transition. This is a variation on theory (1). Whether or not the internal motion observed by crystallography is connected with that observed

*Preliminary measurements⁽¹³⁾ indicated one Gd(III) site on the C_H2 and one on the C_H3 domain. More careful recent studies⁽¹⁾ have shown that both are in fact located on C_H3 .

by PRE is at the present time an unanswered question. In any case more recent work^(17, 18) would seem to provide evidence against the flexible $\rightarrow$ rigid transition idea. For reasons apparent later our studies carried out in the presence of antigen and described below do not provide direct evidence for or against the flexible $\rightarrow$ rigid theory.

Paper II describes PRE studies on a number of Gd(III) $\cdot$ antibody fragment complexes. As experimental conditions could be arranged so that only the C_H3 domain Gd(III) sites were monitored in IgG, the three species IgG, Fc and pFc⁻ form a family of macromolecules with identical Gd(III) binding properties but a range of molecular weights; 150,000 (IgG), 50,000 (Fc) and 25,000 (pFc⁻). The possibilities for a meaningful comparison of PRE parameters in such a system are, in principle, good. In practice however, large errors in the parameters along with the internal motion postulated for IgG limit the usefulness of such comparisons although still allowing some to be made as discussed in paper II.

PRE of pig antibodies in the presence of antigen

The next phase in the PRE/antibody studies was an investigation in the presence of antigen. The major problem here is choosing an antigen-antibody system in which the following conditions can be met as far as possible: (1) a specific antibody must be used - the studies described thus far were carried out on non-immune rabbit IgG (2) the antigen should not bind the paramagnetic ion (Gd(III)) to any significant extent (3) when antibodies and antigens meet there is a great tendency for precipitation: problems arising from this source should be minimised.

In the system we chose to investigate in paper V the antibody was either an early or late population^{*} pig anti-dinitrophen

^{*}'Early' and 'late' refer to the time when these populations occur in the immune response.

(Dnp) antibody and the antigen was a (14-)-Dnp- or mono-Dnp-dextran of variable molecular weight. In the presence of antigen at equivalence ((antigen Dnp groups): (antibody combining sites) $\sim$ 1) the early and late population antibodies are roughly equally efficient in activating complement. However at equivalence the early population antibody (henceforth referred to as precipitating antibody) shows extensive precipitation whereas the late population antibody (non-precipitating antibody) shows none. At the concentrations used in the PRE experiment (some two orders of magnitude greater than for complement fixation assays) we still cannot approach equivalence even for the non-precipitating antibody without some precipitation. It is however desirable to employ as low antigen/antibody ratios as possible as it is known that at high antigen excess complement activation is very inefficient. The non-precipitating antibody allows us to come to a lower antigen/antibody ratio than the precipitating antibody (which can be regarded as the more 'normal' antibody) before precipitation becomes noticeable.

The multivalent antigen used was a 80,000 molecular weight dextran with 14-Dnp groups attached. The monovalent antigens had molecular weights of 10, 20 and 40 $\cdot 10^3$ - unlike the multivalent antigen these were of course not capable of initiating complement activation. None of these Dnp antigens were found to bind Gd(III) to any significant extent.

Old sayings like "if rabbits could fly" and "they breed like pigs" show an awareness of the difference between these animals. Paper V illustrates another difference. Using PRE as a titration indicator, it is found that for pig IgG the Gd(III) binding constants for the Fab and Fc sites are much more similar than for rabbit IgG. Thus, under typical experimental conditions, Gd(III) binding to both sites will be significant. If we are only interested in the yes/no answer to the question of changes in antibody structure on

antigen binding this is a favourable feature - effectively we have a double probe.

For the non-precipitating pig antibody/Gd(III) complex the essential finding of paper V was that there was no change in water proton relaxation behaviour on addition of multivalent antigen. Changes were observed for the precipitating antibody but these were suggested to arise from precipitation effects (although see paper V for a discussion of changes at very high antigen excess). No changes were observed for either antibody in the presence of monovalent antigens.

The results on pig antibody would therefore seem to be consistent with theory (2) of complement triggering. However the large differences in conditions between the PRE experiment and complement fixation studies should be borne in mind. Nevertheless it is striking that the complexation of pig antibody with a large antigen molecule produces no changes detected by the Gd(III) PRE probes. It is tempting to speculate that the domain structure of the antibody molecule serves not to pass "messages" along the molecule but, in a sense, the reverse - to clearly separate the units of function of the molecule.

THE INTERACTION OF PROTEIN A AND Fc AND SIGNIFICANCE FOR COMPLEMENT TRIGGERING

Protein A is isolated from the bacteria staphylococcus aureus where it is found covalently bound to the bacterial cell wall. It binds to the Fc region of normal IgG from several species resulting often in the formation of precipitates. The interaction of protein A with antibody under appropriate conditions results in triggering of the complement sequence making it of considerable interest.

Protein A is an elongated protein of molecular weight 43,000. It has more than one binding site for Fc and is therefore able to cross-link IgG molecules. In fact partial tryptic digestion of protein A yields several low molecular weight fragments (6-8,000) which are monovalent in their binding to the Fc fragment. These fragments originate from four highly homologous Fc-fragment binding regions consecutively arranged in the polypeptide chain of protein A.

Paper IV describes the use of a combination of physical techniques (a) to study the mode of interaction of protein A and one of its tryptic fragments with the Fc fragment and (b) to attempt to detect any conformational changes occurring on interaction. Rabbit Fc was used as, unlike Fc from a number of other species, it forms soluble aggregates with protein A. The physical studies were supplemented by studies on the ability of protein A and one of its fragments to initiate complement fixation with rabbit IgG. Here we shall concentrate on results of significance for the mechanism by which protein A interaction with antibody triggers complement, dealing only very briefly with other aspects.

High resolution proton nmr of protein A gave further evidence for the elongated nature of the molecule although indication of regions of ordered structure involving particularly aromatic residues was found. Proton NMR of the tryptic monovalent fragment VI" again indicated a largely disordered structure and all the aromatic resonances could in this case be assigned.

The interactions of protein A and fragment VI" with the Fc fragment were studied by ultracentrifugation and fluorescence. The former confirmed that fragment VI" does not cause cross-linking of Fc molecules and the latter showed the Fc-protein A interaction to be very strong.

Complement activation studies indicated that protein A in interaction with rabbit IgG did lead to C1 binding to the antibody. The monovalent fragment VI" did not however show this ability under similar conditions. This is of course reminiscent of the situation with antigen-induced complement activation. The monovalent fragment like the monovalent antigen is ineffective in triggering complement. Only the multivalent species - protein A or a multivalent antigen - with the ability to cross-link antibody molecules are effective.

High resolution proton NMR studies of the protein A - Fc fragment suggested that it is the structured regions of the protein A molecule containing most of the aromatic residues which are involved in binding to Fc. These regions are separated in protein A by regions of considerable mobility - a situation which appears to be retained in protein A - Fc fragment complexes. It proved very difficult to draw any conclusions about the effect of complex formation on the Fc fragment because the relatively broad spectral lines of Fc were not easily recognised among the sharper protein A resonances. The situation was compounded as the Fc resonances appeared to be further broadened on complex formation. To avoid this difficulty proton NMR studies were carried out on the Fc-fragment VI" complex. These supported an earlier suggestion that the lone tyrosine of fragment VI" may be involved in binding to Fc. However the most interesting feature to emerge was the marked overall resemblance of the spectrum of the complex to that of Fc alone indicating that no large conformational changes occur in the Fc fragment on the binding of fragment VI".

No changes were found either in the water proton relaxation behaviour (admittedly at only one frequency) of the rabbit Fc fragment/Gd(III) complex on addition of protein A up to stoichiometry.

The lack of changes in the Fc fragment detected by high resolution NMR or PRE on interaction of Fc with protein A or fragment VI", coupled with the inability of the monovalent fragment VI" to trigger complement with rabbit IgG, lead us to propose that triggering by protein A may simply be a result of its ability to cross-link Fc fragments. This is analogous to theory (2) of antigen-induced complement triggering and there would therefore seem to be a pleasing consistency in our results on pig antibodies and the present results.

METAL ION NMR TO PROBE POLYNUCLEOTIDE STRUCTURE AND INTERACTIONS

Nuclear magnetic quadrupole relaxation

As the results in this section are based on the relaxation behaviour of the quadrupolar metal ion nuclei ^{23}Na , ^{25}Mg , and ^{43}Ca , a brief consideration of nuclear magnetic quadrupole relaxation will be presented, in particular that for a spin 3/2 nucleus such as ^{23}Na (reviewed^(19, 20)).

For any nucleus with a spin quantum number (I) greater than 1/2 the distribution of charge over the nucleus deviates from spherical symmetry, a deviation which can be described in terms of a nuclear electric quadrupole moment (eQ). If the electron distribution about the nucleus has less than cubic symmetry then an electric field gradient at the nucleus will result which can couple to the nuclear quadrupole moment. Fluctuations in this field gradient coupled to the quadrupole moment can provide an efficient mechanism for nuclear magnetic relaxation, so efficient that for most quadrupolar nuclei it is the only relaxation mechanism that need be considered.

For an aqueous solution of say NaCl, the ^{23}Na longitudinal and transverse relaxation rates are given by⁽²¹⁾:

$$\tau_1^{-1} = \tau_2^{-1} = \frac{2\pi^2}{5} v_Q^2 \tau_c \quad (1)$$

where $v_Q = e^2 Qq/h$ is the quadrupole coupling constant and τ_c is the correlation time characterising the fluctuations of the electric field gradient. This equation is only valid if τ_c is much shorter than the inverse of the nuclear magnetic resonance frequency, ω^{-1} (so called extreme narrowing conditions, $\omega\tau_c \ll 1$). For $^{23}\text{Na}^+$ in aqueous solution τ_c , believed to involve rotation of water molecules within the hydration sphere of the ion⁽²⁰⁾, is of the order of $10^{-11} - 10^{-12}$ s so that the extreme narrowing condition is easily fulfilled at accessible frequencies.

The binding of $^{23}\text{Na}^+$ to a macromolecule will generally lead to an increase in both τ_c and the field gradient resulting in large increases in relaxation rates. The extreme narrowing condition may no longer be fulfilled when the decays of both longitudinal and transverse magnetisations will, in principle, be non-exponential - in fact different transition probabilities between different nuclear spin states will lead to biexponential decays of the magnetisations. In practice for ^{23}Na , relaxation of the bound ion is not observed directly as the ion normally exchanges sufficiently rapidly so that the observed relaxation is a population weighted average of relaxation in the free and bound states. "Sufficiently rapidly" means that the ions exchange at a much greater rate than the rate of decay of magnetisation in the bound state - this is usually referred to as fast exchange. For other ions e.g. $^{25}\text{Mg}^{2+}$ this fast exchange condition may not be satisfied and the contribution to the overall relaxation from the bound nuclei smaller. Generally a separate signal from the bound quadrupolar nuclei is not observed as relaxation is so efficient as to render the signal extremely broad and therefore not detectable.

Bull⁽²²⁾ has derived approximate equations for the relaxation of an exchanging spin 3/2 nucleus under non-extreme narrowing conditions when the deviation from extreme narrowing is not too great i.e. $\omega\tau_c \lesssim 1.5$. The decay of magnetisations are then approximately exponential and can be described by a single T_1 and single T_2 . For fast exchange conditions the differences in relaxation rates in the presence (T_1^{-1}) and absence (T_{10}^{-1}) of macromolecule can be written as

$$(T_1^{-1})_{\text{ex}} = T_1^{-1} - T_{10}^{-1} = \frac{2\pi^2}{5} p_B \nu_Q^2 \tau_c \left(\frac{0.8}{1+4\omega^2 \tau_c^2} + \frac{0.2}{1+\omega^2 \tau_c^2} \right) \quad (2)$$

$$(T_2^{-1})_{\text{ex}} = T_2^{-1} - T_{20}^{-1} = \frac{\pi^2}{5} p_B \nu_Q^2 \tau_c \left(0.6 + \frac{0.6}{1+\omega^2 \tau_c^2} + \frac{0.4}{1+4\omega^2 \tau_c^2} + \frac{0.4}{1+\omega^2 \tau_c^2} \right) \quad (3)$$

where p_B is the fraction of nuclei bound to the macromolecule. The equations as presented assume only one type of macromolecule binding site but are easily extended to include several. From these equations it is seen that (a) if $\omega^2 \tau_c^2 \ll 1$, equation (1) is obtained again ($p_B = 1$) (b) if $\omega^2 \tau_c^2 \gtrsim 1$, τ_c can be obtained either from the ratio T_1/T_2 or the frequency dependence of T_1 or T_2 and (c) only the product $p_B \nu_Q^2$ can be obtained from relaxation measurements.

The decays of magnetisation for the spin 5/2 (eg. Mg^{2+}) and 7/2 (eg. Ca^{2+}) will also be non-exponential when non-extreme narrowing conditions apply. These cases have been considered in detail elsewhere⁽²³⁾.

²³Na NMR to probe ion binding to chromatin

Virtually all the DNA of higher organisms is found in the

cell nucleus in the form of chromatin^x. The DNA is greatly compacted in chromatin largely by virtue of its interaction with a group of proteins known as histones. The histones contain a large number of positively charged basic amino acid residues (lys, arg) which can interact with the negative phosphate groups of DNA. However there is far from exact mutual neutralisation between these two species in chromatin e.g. it is estimated that only approx. 60 % DNA phosphates are neutralised by histone basic residues the rest being taken up typically by Na^{+} ⁽²⁴⁾. Clearly Na^{+} ions have an important role to play in the chromatin structure.

In paper VII we attempt to learn something about this role by using ^{23}Na NMR to compare Na^{+} binding to DNA in "free" DNA and chromatin. The approach adopted was to study ^{23}Na relaxation behaviour of calf thymus chromatin in increasing NaCl concentration corresponding to an increased dissociation of histones from DNA. It was found that the T_1/T_2 ratio for ^{23}Na relaxation decreased markedly on going from chromatin (low salt concentration) to the free species, DNA and histones (high salt). This result correlated well with a similar effect observed for urea dissociation of chromatin. The differences in T_1/T_2 were traced to differences in τ_c (see equations (2, 3) and paper VII) - τ_c was longer for binding to the chromatin complex than to the free species.

With the reasonable assumption that Na^{+} binding to histones is negligible compared to that to DNA, we were then able to conclude the existence of Na^{+} binding sites in chromatin of a different nature to those found in DNA.

Although not affecting our main conclusion, it is of interest to consider our results in the light of the possible nature of τ_c , which is given by the equation

^xThis applies strictly to the "resting state" of the cell. In anticipation of and during cell division the chromatin becomes organised into distinct linear structures - the chromosomes.

$$\tau_C^{-1} = \tau_R^{-1} + \tau_M^{-1} \quad (4)$$

where τ_R is a rotational correlation time and τ_M the lifetime of the ion in the bound state. If $\tau_C = \tau_R$ then our results reflect restricted motional freedom of Na^+ sites in chromatin; if $\tau_C = \tau_M$ then a decreased access of Na^+ to binding sites in chromatin is implied.

Future chromatin work using ^{23}Na NMR is planned on nucleosomes and core particles when the kind of questions we will be asking are:

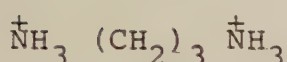
- (1) Are there any differences in the nature of Na^+ sites in chromatin as used by us above and nucleosomes?
- (2) Are there any differences between Na^+ sites in the core particle and linker regions of the nucleosome?
- (3) What effect does removal of the "tails" of the histones in the nucleosome have on Na^+ binding?
- (4) What is the relationship between divalent ion e.g. Mg^{2+} and monovalent ion e.g. Na^+ binding to chromatin?

Polyamine binding to DNA studied by ^{23}Na NMR

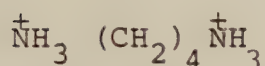
Polyamines (for a review see ⁽²⁵⁾) are ubiquitous in biological materials although the relative amounts of the principal polyamines putrescine, spermidine and spermine differ markedly in different cells. Prokaryotes in general have a higher concentration of putrescine than spermidine and lack spermine whereas eucaryotes usually have little putrescine and have spermine as well as spermidine. Interestingly the bacteriophage T4 and the Herpes simplex virion are found to contain enough polyamine to neutralise roughly half of the viral DNA in each case ^(26, 27). Among the many known biological functions of polyamines can be mentioned their ability to stabilise polynucleotide structures, to stabilise membranes, to stimulate DNA and RNA synthesis in vitro and

to stimulate protein synthesis in vitro and in vivo. The specific mechanisms by which polyamines fulfill these physiologically important roles remain largely unknown.

We have been interested in the interaction of polyamines with DNA, an interaction which can be compared to that of DNA with the histones since the polyamines are also heavily positively charged species at neutral pH as shown below:



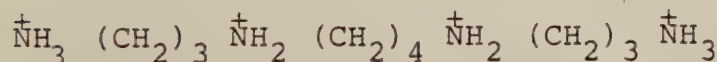
1,3 diaminopropane



1,4 diaminobutane (putrescine)



spermidine



spermine

Using ^{23}Na NMR to attempt to obtain quantitative information on the binding of polyamines to DNA, we have titrated DNA with the various polyamines following the course of the titration by the decreased ^{23}Na linewidth as the polyamines compete with Na^+ ions for the DNA phosphate groups. Fig. 3 illustrates a comparison of titrations for 1,3 diamino-propane, 1,4 diaminobutane and Mg^{2+} . The curve for the two polyamines show a number of interesting features. In the first instance both bind with a much greater affinity than Na^+ so that the stoichiometry of the interaction with DNA can be determined: the intercept of 0.5 corresponds to one polyamine N^+ group per DNA phosphate group.

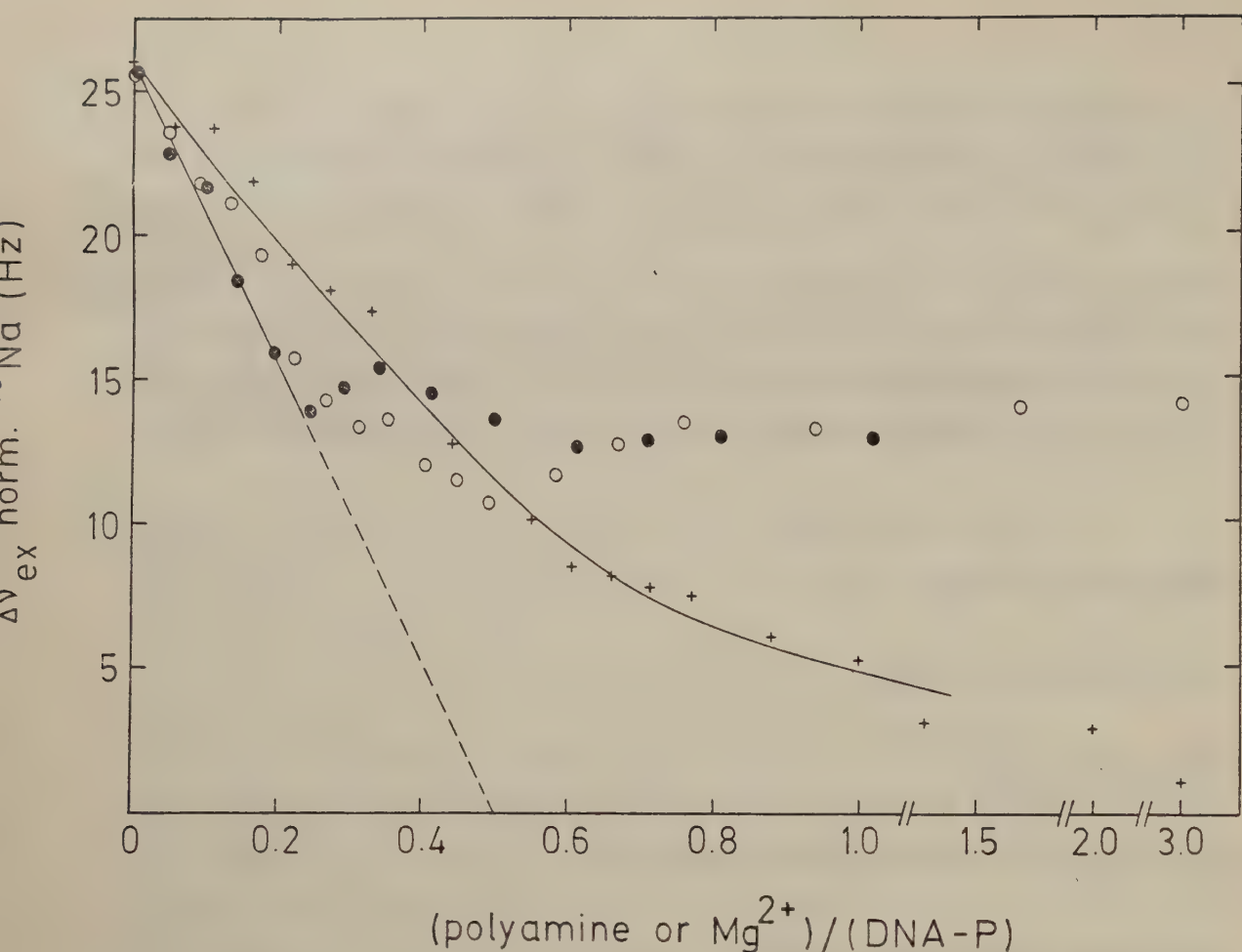


Fig. 3. The normalised ^{23}Na excess linewidth as a function of added diaminopropane (●), diaminobutane (o) and Mg^{2+} (+) for an aqueous solution of DNA.

The concentration of Na^+ (sodium phosphate) in each case was 45 mM; the concentration of DNA in phosphate, (DNA-P), in each case was within 2 mM of 16 mM and all relaxation rates are normalised to this concentration; pH, 6.8; temperature, $4 \pm 1^\circ\text{C}$.

The curves give no indication of cooperativity. A remarkable feature is that the curves level off at the same value corresponding to a reduction of $\sim 50\%$ in the excess ^{23}Na linewidth. The most ready interpretation of this is that a saturation point is reached when 50 % of the DNA phosphates are neutralised by either of the divalent

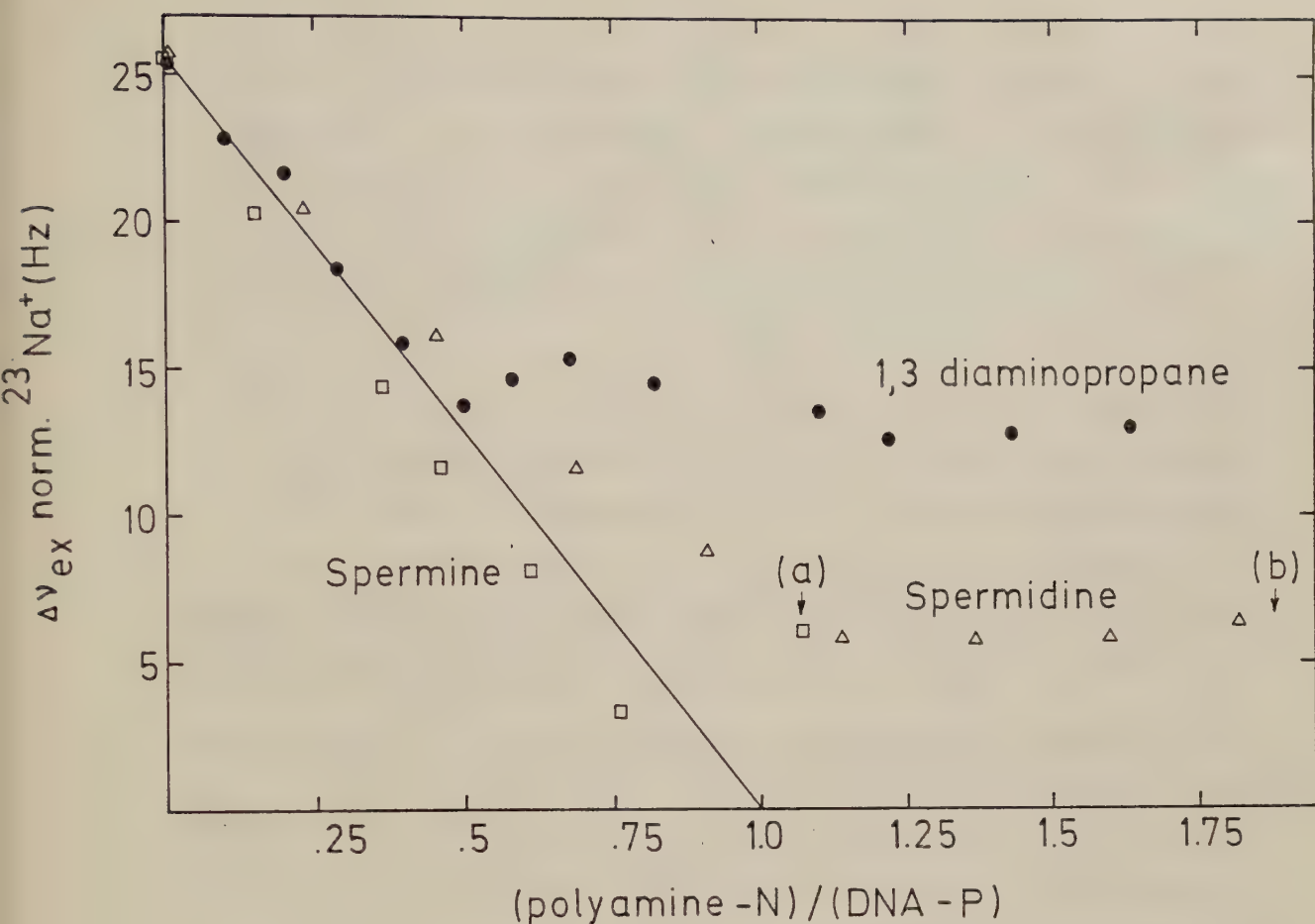


Fig. 4. The normalised ^{23}Na excess linewidth as a function of added diaminopropane (o), spermidine (Δ) and spermine ($\square$).

Conditions as in Fig. 3 except that the concentration of DNA in phosphate for the spermine case was only 4 mM as precipitation problems are more acute at higher DNA concentrations. (a) and (b) indicate the onset of precipitation for spermine and spermidine respectively.

polyamines. The middle regions of the binding curves are complex and will not be taken up in this brief discussion. Fig. 3 also shows that Mg^{2+} binds with a weaker affinity than the polyamines, displaces virtually all the Na^+ ions from the DNA surface and binds with a stoichiometry corresponding roughly to one Mg^{2+} ion per DNA phosphate group.

Fig. 4 compares titrations for the polyamines 1,3 diaminopropane (2 amino groups), spermidine (3) and spermine (4). An extrapolated line drawn to intercept the abscissa at a (polyamine N): (DNA-phosphate) ratio of 1.0 reproduces reasonably well the initial portions of the 3 curves indicating that all 3 polyamines have a much greater affinity for DNA than Na^+ and that in each case one polyamine N^+ group binds per DNA phosphate group. Spermidine shows a levelling off feature as for 1,3 diaminopropane but at $\sim 25\%$ of the total excess ^{23}Na linewidth as compared to $\sim 50\%$ for the latter. The situation for spermine at higher spermine concentrations is unclear because of the onset of precipitation.

In this brief discussion we have avoided any detailed interpretations of the results. These must await further experimental work which when coupled with model building studies could provide us with much information on the DNA-polyamine interaction.

The melting of DNA and transfer RNA

The thermal denaturation of DNA or transfer RNA (tRNA) is often referred to as melting. The course of melting is generally followed by observing the increase in UV absorption at 260 nm as double helical base stacking interactions are lost (the hyperchromic effect). In Fig. 5 we have followed the melting of DNA in the presence of Ca^{2+} using ^{43}Ca NMR. At the lower temperatures there is a steady increase in the ^{43}Ca linewidth because, presumably, the linewidth is then determined by chemical exchange. At roughly 77°C the linewidth shows a very pronounced discontinuity which correlates well with the melting temperature (T_m) expected for calf thymus DNA under the experimental conditions used. The abrupt nature of the discontinuity reflects the highly cooperative nature of the melting process.

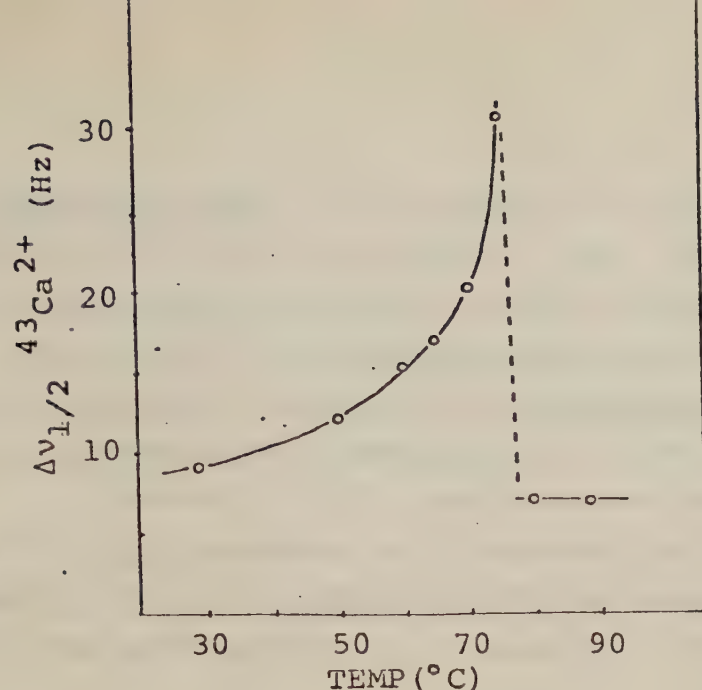


Fig. 5. The ^{43}Ca linewidth in an aqueous solution of DNA as a function of temperature.

The Ca^{2+} concentration was 41 mM (60% enriched in $^{43}\text{Ca}^{2+}$); the DNA concentration 5 mM in phosphate; 10 mM tris/ Cl^- buffer, pH 7.0.

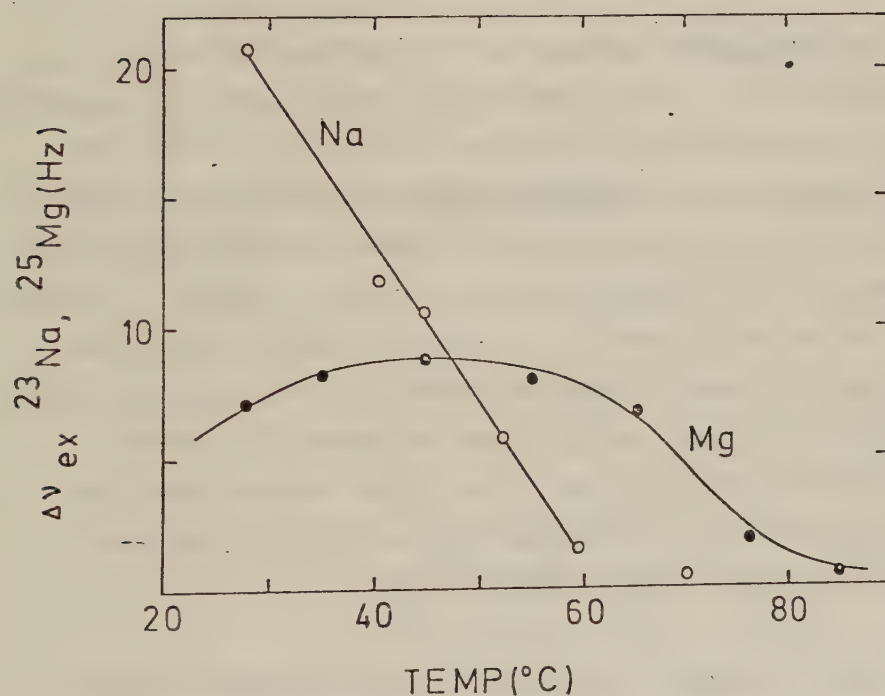


Fig. 6. The ^{23}Na and ^{25}Mg excess linewidths in an aqueous solution of tRNA as a function of temperature.

○, ^{23}Na . The concentration of Na^+ (NaCl) was 30 mM; the tRNA concentration 8.1 mM in phosphate; 10 mM tris/ Cl^- buffer; pH 7.4.

●, ^{25}Mg . The concentration of Mg^{2+} (MgCl_2) was 0.1 M (90% enriched in $^{25}\text{Mg}^{2+}$); the tRNA concentration 12.4 mM in phosphate; 10mM tris/ Cl^- buffer, pH 7.9.

Fig. 6 shows the thermal denaturation of tRNA followed by ^{23}Na and ^{25}Mg NMR. The ^{23}Na excess linewidth exhibits a temperature dependence characteristic of fast exchange conditions at all temperatures whereas the ^{25}Mg excess linewidth exhibits a maximum indicative of changes in the Mg^{2+} binding sites on tRNA as the temperature is increased. At lower temperatures ($<45^{\circ}\text{C}$) chemical exchange would seem to dominate ^{25}Mg relaxation while the large decrease in linewidth in the region $65 - 80^{\circ}\text{C}$ would seem to correspond to changes in tRNA structure in this region.

No further discussion of the above results will be presented here - they are included simply to illustrate the sensitivity of metal ion NMR to changes in polynucleotide structure, here exemplified by the melting process.

Conclusions on metal ion NMR in polynucleotide systems

A comment which has been passed as regards chromatin is that we know a fair deal about the structure of chromatin but very little about what the ions are doing. We have shown that ^{23}Na NMR can provide information on the binding of Na^{+} to chromatin. The binding of polyamines to DNA is poorly understood on a quantitative level. Using ^{23}Na NMR we are able, relatively easily, to obtain quantitative binding information and it seems possible that the results could ultimately be used to propose a model for DNA-polyamine interactions. Finally we have shown how the melting of DNA and tRNA is reflected in the relaxation behaviour of ^{43}Ca and ^{25}Mg respectively.

Clearly the work on metal ion NMR is in its preliminary stages but the above examples hopefully illustrate the richness of the possibilities in this area.

ACKNOWLEDGEMENTS

I should like to thank

- above all Sture Forsén for many things, both scientific and personal, but more than anything for the kindness and thoughtfulness he has shown me.

- everyone in the department with a special thanks to Tom Bull, Björn Lindman and Håkan Wennerström and my mates, Pétur and Thomas.

- Raymond Dwek, for an enormous amount of help and for many pleasant times in Oxford, and also everyone in Raymond's group.

- the following (in random order) Alan McL., Inger A., Gunilla I-F., Sven L., Calle B., Maurice G., Gil N., Seymour K., Iain C., Kay D., Ian W., Sue and Hugo., Bruce S., Eileen, Skim, Ada T., Jiri N., Eva H., Bodil P., Maria L., Joseph P., Sven-Åke P., Kurt V., Simon W-H, Frantisek F., Jörgen S., Folke T. and Keith W.

Finally an extra special thanks to Alice, Damian and my parents.

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